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Channel γ Subunit Expressed in Differentiating Neurons and Myoblasts

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Transient elevations of intracellular calcium (calcium transients) play critical roles in many developmental processes, including differentiation. Although the factors that regulate calcium transients are not clearly defined, calcium influx may be controlled by molecules interacting with calcium channels, including channel regulatory subunits. Here, we describe the chick γ4 regulatory subunit (CACNG4), the first such subunit to be characterized in early development. CACNG4 is expressed early in the cranial neural plate, and later in the cranial and dorsal root ganglia; importantly, the timing of this later expression correlates precisely with the onset of neuronal differentiation. CACNG4 expression is also observed in nonneuronal tissues undergoing differentiation, specifically the myotome and a subpopulation of differentiating myoblasts in the limb bud. Finally, within the distal cranial ganglia, we show that CACNG4 is expressed in placode-derived cells (prospective neurons), but also, surprisingly, in neural crest-derived cells, previously shown to form only glia in this location; contrary to these previous results, we find that neural crest cells can form neurons in the distal ganglia. Given the proposed role of CACNG4 in modulating calcium channels and its expression in differentiating cells, we suggest that CACNG4 may promote differentiation via regulation of intracellular calcium levels.

Key Words: **CACNG4 (γ4); calcium channel; neuronal differentiation; myoblasts; cranial ganglia; placodes.**

INTRODUCTION

Calcium is an important second messenger in a wide variety of biological processes, including neurotransmission, muscle contraction, growth factor signaling (Kühl *et al.*, 2000), and regulation of gene expression (Ginty, 1997). In development, calcium signaling has likewise been shown to play many different roles at different stages. Transient elevations of intracellular calcium levels (calcium transients) have been observed in early embryos of many species, including zebrafish (Creton *et al.*, 1998; Gilland *et al.*, 1999; Webb and Miller, 2000) and *Xenopus* (Leclerc *et al.*, 2000; Wallingford *et al.*, 2001); experimental blockage of these transients at various stages can prevent cytokinesis (reviewed by Webb and Miller, 2000), block convergent extension morphogenetic movements during gastrulation (Wallingford *et al.*, 2001), and impair neural induction (Leclerc *et al.*, 2000).

At later stages, calcium has been shown to be an important factor in differentiation. This has perhaps been demonstrated most clearly in cultured embryonic *Xenopus* spinal neurons, which exhibit two types of spontaneous calcium transients, spikes and waves (reviewed by Gu and Spitzer, 1997). When these transients are blocked, neuronal differentiation is altered; furthermore, under these conditions, production of spikes is sufficient to induce neurotransmitter expression and channel maturation, while production of waves can promote neurite extension (Gu and Spitzer, 1995). Thus, not only are calcium transients necessary and sufficient for differentiation, but changes in the kinetics of the transients can affect distinct aspects of differentiation. Interestingly, spikes and waves are also observed in neural crest cells undergoing neurogenesis in culture, and blockage of the calcium transients reduces the number of differentiated neurons formed (Carey and Matsumoto, 1999), suggesting a conserved role for transients in neuronal differentiation in both the central and peripheral nervous systems. However, calcium transients are also observed in nonneural differentiating cells, including em-

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bryonic *Xenopus* myocytes; blockage of these transients disrupts myofibrillogenesis and somite maturation (Ferrari *et al.*, 1996; Ferrari and Spitzer, 1999).

Given the developmental significance of calcium transients, it is important to determine what molecules regulate these transients during development. Transients can be generated by release of calcium from intracellular stores or by influx of extracellular calcium through channels. Calcium-permeable channels include NMDA and AMPA glutamate receptors, but the main class is voltage-dependent calcium channels (VDCCs), which mediate calcium entry in response to membrane depolarization. VDCCs are composed of four subunits: α_1, β, $\alpha_2\delta$, and γ. α_1 forms the pore of the calcium channel and can alone mediate calcium entry; thus, the additional subunits play accessory/regulatory roles (Walker and De Waard, 1998). γ specifically has been shown to alter peak current as well as activation and inactivation kinetics (Singer *et al.*, 1991; Wei *et al.*, 1991; Eberst *et al.*, 1997; Freise *et al.*, 2000).

The first γ subunit (γ1) was isolated from skeletal muscle, and the protein was detected in skeletal muscle only (Jay *et al.*, 1990). However, a highly divergent γ gene (γ2, or stargazin) was later identified based on its disruption in the stargazer mouse (Letts *et al.*, 1998). Stargazer mutants display neurological defects characteristic of absence epilepsy; accordingly, γ2 expression was only detected in the brain. Functionally, γ2 was shown to alter inactivation kinetics of VDCCs (Letts *et al.*, 1998); thus, it was proposed that γ2 has the same general role as γ1 in modulating VDCC activity. However, it has subsequently been proposed that γ2 acts instead via AMPA glutamate receptors (Chen *et al.*, 2000; see Discussion). Six additional γ subunit genes, CACNG3–8 (CAlcium ChaNnel Gamma 3–8), have since been isolated from humans (Burgess *et al.*, 1999, 2001) and mice (Klugbauer *et al.*, 2000). However, the functional characteristics and expression patterns of these genes have not been extensively studied, nor have the expression patterns for these or any γ subunits been examined during development.

Although signaling by calcium transients has been shown to be important throughout development, there is little known about the molecules that mediate or regulate these transients. Here, we describe the chick homologue of CACNG4, the first γ subunit thereby shown to be expressed during early development. We show that CACNG4 is expressed in multiple neural tissues during initiation of neuronal differentiation, as well as in differentiating myoblasts. Surprisingly, we also find expression of CACNG4 in cells of neural crest origin in the distal cranial ganglia; although these cells were previously thought to form only glia in this location, we show that neural crest cells can indeed give rise to neurons in the distal ganglia. In conclusion, we suggest that CACNG4 plays a general role in differentiation, perhaps involving regulation of calcium transients.

MATERIALS AND METHODS

Manipulation of Avian Embryos

Fertilized chick and quail eggs were obtained from AA Laboratories, (Westminster, CA) and incubated at 38°C in humidified, rocking incubators (Lyon Electric Company, Chula Vista, CA). Embryos were staged according to Hamburger and Hamilton (1951) (HH). For labeling of placodal cells, the ectoderm lateral to the hindbrain was mechanically excised from four- to seven-somite quail embryos by using pulled glass needles and grafted into the same location in similarly staged chick embryos, as described in Baker *et al.* (1999). For labeling of neural crest cells, the dorsal neural folds of the hindbrain were bilaterally excised from 8- to 10-somite quail embryos and grafted into chick embryos as described in D'Amico-Martel and Noden (1983). All quail/chick grafts were incubated for 48 h, until approximately stage 20.

Isolation of CACNG4

D6U3-3, a 121-bp PCR fragment generated from a differential display screen (Martinsen and Bronner-Fraser, 1998), was cloned into pGEM-T (Promega); the sequence of this insert was obtained by using the ABI Prism DNA Sequencing Kit (Perkin-Elmer Applied Biosystems) and an automated sequencer. To obtain additional 5′ sequence, 5′ RACE (Rapid Amplification of cDNA Ends) was performed, using primers designed from D6U3-3 and a 5′ RACE kit from Roche Molecular Biochemicals. The resulting 303-bp fragment was joined with the previous plasmid to generate a 424-bp probe, which was subsequently used to screen, using standard techniques (Sambrook *et al.*, 1989), an unamplified cDNA lambda phage library prepared from HH stage-8 to -10 poly(A) mRNA (Kee and Bronner-Fraser, 2001). One positive clone was obtained and sequenced. BLAST analysis, using the basic BLAST search facility at NCBI (www.ncbi.nlm.nih.gov/BLAST/), indicated strong homology to several calcium channel γ subunits, and indicated that the clone was incomplete at the 5′ end. Therefore, 5′ RACE was again performed, using a kit from Gibco/BRL, and the resulting 370-bp fragment was ligated into the previous plasmid to generate a complete open reading frame of 987 bp. Sequences were analyzed by using DNAStar software and the TMPred program for prediction of transmembrane regions (Hofmann and Stoffel, 1993; www.ch.embnet.org/cgi-bin/TMPRED_form.html).

In Situ Hybridization and Immunohistochemistry

Whole-mount *in situ* hybridization was performed as described by Wilkinson (1992), using an antisense RNA probe against the entire CACNG4 coding region. For most sections shown (see Figs. 2 and 3), *in situ* hybridization was performed on whole embryos, which were subsequently dehydrated, embedded in paraffin, and sectioned at 20 μm on a Leitz microtome. Double *in situ* hybridization for CACNG4 and myoD was performed as described in Knecht *et al.* (1995), with the following notes. Antisense RNA probes were labeled with digoxigenin-UTP for CACNG4, and fluorescinated UTP for myoD. Hybridization of both probes was performed simultaneously by using the procedure of Wilkinson (1992). CACNG4 was visualized first, using the anti-digoxigenin antibody and NBT/BCIP substrates, as described; myoD was subsequently stained by using the anti-fluorescein antibody (diluted 1:5000) and INT/BCIP substrate (stock solution of 33 mg/ml, diluted 1:286) (Roche Molecular Biochemicals).

TABLE 1
Percent Similarity between Subunits

	Human gamma 4	Mouse gamma 4	Mouse gamma 2	Mouse gamma 1
Chick γ 4	87.8	86.9	59.1	12.1
Human γ 4		98.2	55.1	13.9
Mouse γ 4			55.1	13.9
Mouse γ 2				16.1

For CACNG4 *in situ* hybridization/QCPN antibody staining of quail/chick chimeras (see Fig. 4), *in situ* hybridization was performed directly on 6-μm paraffin sections, generated from embryos fixed in modified Carnoy's solution (60% ethanol, 11.1% formaldehyde, 10% acetic acid). The procedure of Etchevers *et al.* (2001) was followed, with the following modifications: proteinase K was not used, and slides were washed (after hybridization) in 50% formamide, 1× SSC, 0.1% Tween 20. Following staining with NBT/BCIP and fixation in 4% paraformaldehyde, slides were washed in phosphate-buffered saline (PBS) and then incubated for 30 min in 1.2% hydrogen peroxide in PBS. Slides were washed as before and then blocked briefly in PBS containing 0.2% bovine serum albumin, 1% Triton X-100, and 5% heat-inactivated goat serum (PBTG). Undiluted QCPN supernatant (Developmental Studies Hybridoma Bank) was added to slides, under coverslips, and slides were incubated overnight at 4°C in a humid chamber. For a secondary antibody, we used horseradish peroxidase-conjugated rabbit anti-mouse IgG1 (Zymed), diluted 1:200 in PBTG. Finally, slides were stained for 30 min in 0.05 mg/ml 3–3′ diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich), 0.005% hydrogen peroxide in PBS.

For Hu/QCPN double antibody staining (see Fig. 5), 8-μm gelatin cryostat sections were washed in PBS and blocked briefly in PBTG. Slides were incubated sequentially in: anti-HuC/HuD (Molecular Probes, Eugene, OR), diluted 1:500 in PBTG; biotin-conjugated goat anti-mouse IgG2b (Southern Biotechnology Associates, Inc., Birmingham, AL), diluted 1:100 in PBTG; NeutrAvidin (Molecular Probes), diluted 1:100 in PBS; QCPN supernatant, undiluted; and TRITC-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates, Inc.), diluted 1:200 in PBTG.

RESULTS

Chick Gene Has High Homology to Calcium Channel γ4 Subunits

A 121-bp fragment from the 3′-untranslated region of CACNG4 was initially PCR amplified in a differential

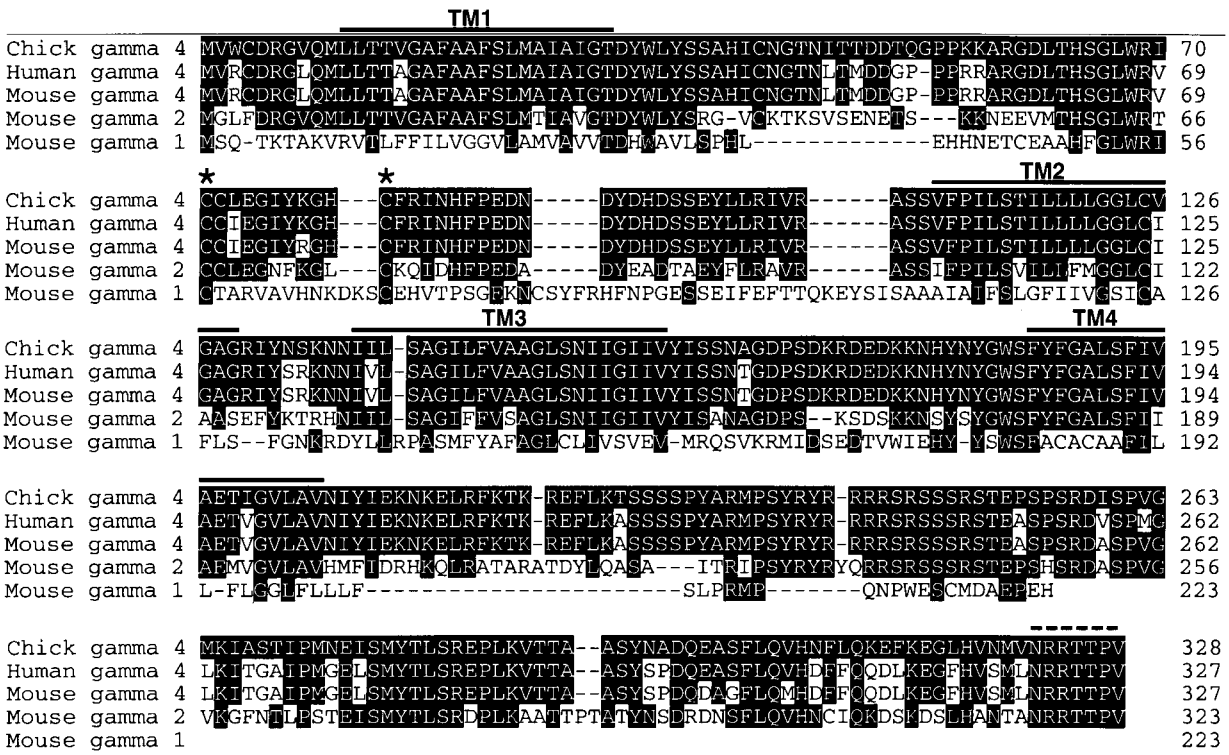

FIG. 1. Sequences of γ subunit genes. Alignment of chick γ4 (CACNG4), human γ4, mouse γ4, mouse γ2 (stargazin), and mouse γ1. Conserved residues are indicated by black shading; percentage similarities are given in Table 1. Solid black bars designate the four transmembrane domains (TM1–4) predicted for chick CACNG4 by the TMPred program (Hofmann and Stoffel, 1993). Asterisks indicate two cysteine residues conserved in all γ subunits. The dashed black line at the C terminus marks a consensus site for cAMP and cGMP-dependent protein kinase phosphorylation, as well as a type I PDZ-binding site.

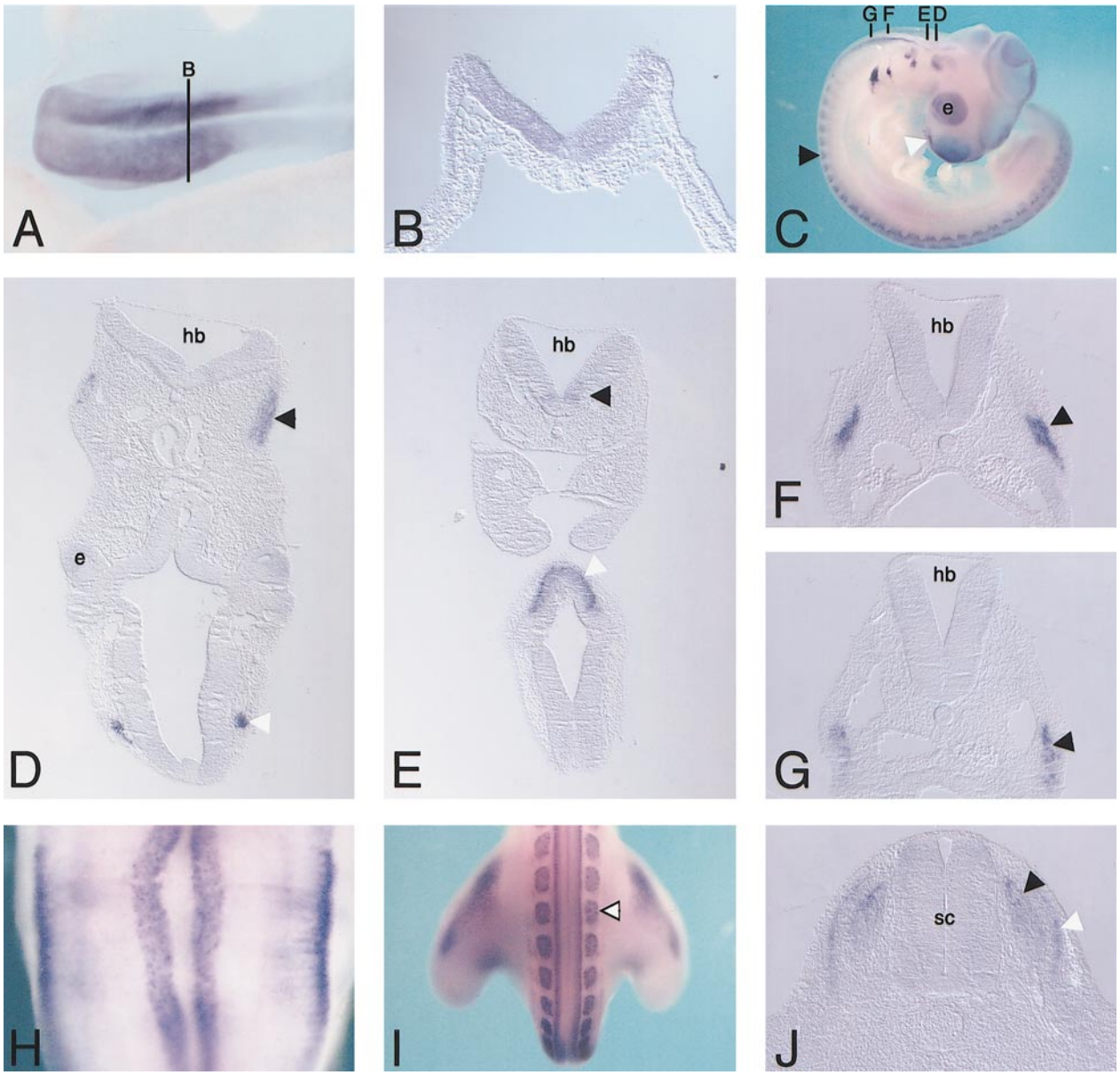

FIG. 2. Expression of CACNG4 in chick embryos. (A) CACNG4 expression (blue) in the neural plate of a three-somite embryo (stage 8). Anterior is to the left. Expression is restricted to the anterior neural plate and excluded from the ventral midline. The line indicates the level of the section shown in (B). (B) Transverse section through the embryo shown in (A), demonstrating CACNG4 expression in the neural plate but not underlying tissues or nonneural ectoderm. (C) Expression of CACNG4 in a 3-day embryo (stage 18), in the cranial ganglia, including the olfactory (white arrowhead), trigeminal, acoustic, geniculate, petrosal, and nodose ganglia, in the hindbrain, and in the dorsal root ganglia of the trunk (black arrowhead). Weak staining in the brain and eye (e) represents background due to trapping. Lines at the top of the figure show the level of subsequent sections as indicated. (D–G) Sections through the head and branchial region of a 3-day embryo (stage 18). (D) Section through the hindbrain (hb) and the ventral regions of the forebrain and eyes. CACNG4 is expressed in the trigeminal ganglia (black arrowhead) under the hindbrain and in the olfactory nerves (white arrowhead). (E) More posterior section through the head, demonstrating CACNG4 expression in the ventral hindbrain (black arrowhead) and in the infundibulum of the ventral diencephalon (white arrowhead). (F) CACNG4 expression in the distal petrosal ganglia (arrowhead). (G) Staining in the distal nodose ganglia (arrowhead). (H) Dorsal view of a slightly flattened hindbrain in whole mount of a 3.5-day embryo (stage 21). CACNG4 is expressed in broad stripes just lateral to the ventral midbrain, as well as thinner, more dorsal stripes. (I) Dorsal view of the tail of a 4-day embryo (stage 23), showing staining in the spinal cord and hindlimb buds, and overlapping staining of the DRGs and myotome (arrowhead). (J) Transverse section through the posterior trunk of a 4-day embryo, with CACNG4 expression in the DRGs (black arrowhead) and myotome (white arrowhead).

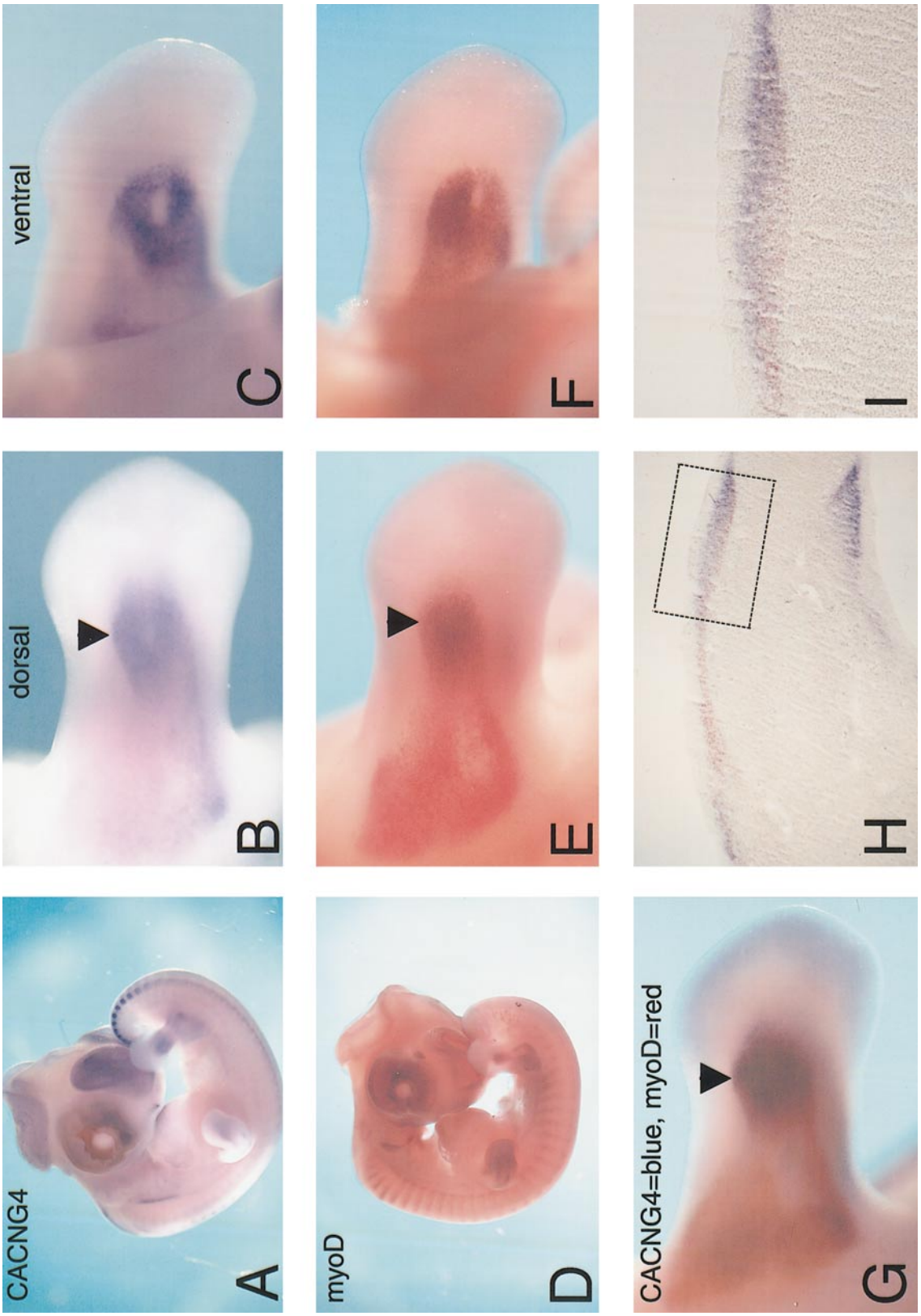

FIG. 3. Expression of CACNG4 in differentiating muscle masses in the limb buds of 5-day embryos (stage 26). Arrowheads indicate distal region of dorsal muscle mass. (A–C) *In situ* hybridization for CACNG4 (blue). (A) Side view, showing CACNG4 expression in the limb buds and in the DRGs/myotome of the tail. (Staining in the telencephalon and eyes is background due to trapping.) (B) Dorsal view of staining in the leg bud. (C) Ventral view of staining in the leg bud. (D–F) *In situ* hybridization for myoD (red). (D) Side view showing myoD expression in the somites and limbs. (E) Dorsal view of staining in the leg bud. (F) Ventral view of staining in the leg bud. (G–I) Double *in situ* hybridization for CACNG4 (blue) and myoD (red). (G) Dorsal view of staining in the leg bud. (H) Transverse section through a double-stained leg bud, as shown in (G), with the distal end toward the right, and dorsal toward the top. CACNG4 is expressed in two stripes, in the dorsal and ventral muscle masses; this expression overlaps that of myoD, but not completely. Box indicates the region enlarged in (I).

display screen (Martinsen and Bronner-Fraser, 1998). A complete clone was subsequently identified through 5′ RACE and library screening; the nucleotide and deduced protein sequences have been submitted to GenBank (Accession No. AY037891). Analysis of the sequence revealed that this gene likely represents the chick homologue of the γ4 (gamma 4, or CACNG4) subunit of voltage-dependent calcium channels, given the high homology between this sequence and those of human CACNG4 (Burgess *et al.*, 1999) and mouse CACNG4 (Klugbauer *et al.*, 2000) (87.8 and 86.9% similarities, respectively; Table 1 and Fig. 1). Chick CACNG4 is also quite similar to mouse γ2 (59.1%), the stargazin gene (Letts *et al.*, 1998), in agreement with previously published molecular phylogenies (Burgess *et al.*, 1999), indicating a close relationship between γ4 and γ2 subunits. In contrast, chick CACNG4 is only 12.1% similar to the mouse γ1 subunit (Wissenbach *et al.*, 1998).

The chick CACNG4 protein is predicted to contain four transmembrane domains (black bars labeled TM1–4, Fig. 1) and intracellular amino and carboxyl termini. The transmembrane topologies predicted for the chick gene match those predicted for human CACNG4 (Burgess *et al.*, 1999) and agree with the proposed structure for γ subunits in general as four-pass transmembrane proteins. Chick CACNG4 additionally contains two cysteines conserved in all γ subunits (asterisks, Fig. 1), which are thought to form a disulfide linkage (Burgess *et al.*, 2001). Also conserved among CACNG2, -3, -4, and -8, are the seven carboxyl-terminal amino acids (dashed line, Fig. 1), which form both a consensus phosphorylation site for cAMP and cGMP-dependent kinases as well as a type I PDZ-binding site (Burgess *et al.*, 2001), which in stargazin is important for activating AMPA receptors (Chen *et al.*, 2000; see Discussion).

CACNG4 Is Expressed Early in the Anterior Neural Plate and Later in Differentiating Neurons

The initial fragment of CACNG4 was isolated based upon its expression in cranial neural folds (at HH stage 8) and its absence in trunk tissue, in a differential display screen described previously (Martinsen and Bronner-Fraser, 1998). Accordingly, whole-mount *in situ* hybridization revealed that CACNG4 is expressed in the neural plate of young stage-8 (1–2 somites) chick embryos, but only in the cranial region (Figs. 2A and 2B). CACNG4 is expressed throughout the cranial neural plate except for the ventral midline, or prospective floor plate. This early pattern of expression disappears by stage 10.

CACNG4 expression is not detected again until stage 14–15, when it appears first in the cranial ganglia. Expression begins in the trigeminal and geniculate ganglia (of cranial nerves V and VII), and progresses posteriorly in accordance with the anteroposterior sequence of development in avian embryos. By stage 18 (30 somites), expression is visible in the developing olfactory nerve (white arrowheads in Figs. 2C and 2D), both lobes of the trigeminal ganglion (Fig. 2C; black arrowhead in Fig. 2D), the acoustic and geniculate ganglia (Fig. 2C), the petrosal ganglion (of cranial nerve IX; Figs. 2C and 2F), and the nodose ganglion (of cranial nerve X; Figs. 2C and 2G). The timing of this expression pattern in the cranial ganglia closely matches that of the Hu proteins, early markers of neuronal differentiation (Wakamatsu and Weston, 1997), suggesting that CACNG4 marks differentiating neurons. Also like Hu, CACNG4 expression is transient. Note that expression in the anterior trigeminal ganglion at stage 18 has already begun to diminish; loss of expression progresses from anterior to posterior until stage 26 (Fig. 3A), when no expression can be detected in the cranial ganglia.

In the brain, CACNG4 is expressed strongly in the infundibulum (prospective posterior pituitary, or neurohypophysis; white arrowhead, Fig. 2E), and weakly in the ventral hindbrain (black arrowhead, Fig. 2E), in cells just dorsal to the floor plate which may represent differentiating motor neurons. These regions also stain strongly for Hu at these stages (data not shown). Expression of CACNG4 in the hindbrain intensifies over time and expands to include a more dorsal stripe of cells by stage 21 (Fig. 2H). Expression also extends posteriorly into the spinal cord, and even into the tail by stage 23 (Fig. 2I).

In the trunk, CACNG4 is also expressed in the dorsal root ganglia (DRGs; black arrowheads, Figs. 2C and 2J; arrowhead, Fig. 2I), beginning at stages 17–18. At these stages, the DRGs are beginning to differentiate as demonstrated by initial expression of Hu (Marusich *et al.*, 1994). Thus, in a variety of neural tissues, CACNG4 expression correlates with the onset of neuronal differentiation.

CACNG4 Is Expressed by Differentiating Myoblasts in the Limb Buds

In addition to its expression in neural tissues, CACNG4 is also expressed in prospective muscle: in the myotome of the somites (overlaps segmental DRG staining in Fig. 2I, arrowhead; white arrowhead, Fig. 2J) and in dorsal and ventral patches in the limb buds (Figs. 3A–3C). These patches strongly resemble the dorsal and ventral muscle masses marked by myoD, a key regulator (and marker) of muscle differentiation (Davis *et al.*, 1987; Pownall and Emerson, 1992) (Figs. 3D–3F). In order to determine whether the pattern of CACNG4 expression overlaps with that of myoD, we performed double *in situ* hybridization for both genes (CACNG4 stained blue, myoD stained red, Figs. 3G–3I) in whole 5-day (stage 26) embryos. These experiments revealed that these genes are expressed in overlapping regions, but not exclusively. For example, both genes are strongly coexpressed in the distal part of the dorsal muscle mass (arrowheads, Figs. 3B, 3E, and 3G; box in Fig. 3H; Fig. 3I), but the red myoD staining extends further ventrally into the limb (Fig. 3I). MyoD is also expressed further proximal to this distal patch, where CACNG4 expression is weak or absent (middle region, Fig. 3H;

compare also Figs. 3B and 3E). We therefore conclude that CACNG4 is expressed by a subset of differentiating myoblasts.

CACNG4 Is Expressed by Cells of Both Neural Crest and Placode Origin

Many of the cranial ganglia are formed from both neural crest cells (LeDouarin and Kalcheim, 1999), which migrate from the dorsal neural tube, and ectodermal placode cells, which invaginate from thickenings in the cranial ectoderm (Baker and Bronner-Fraser, 2001). The contribution of each population to the cranial ganglia has been carefully mapped, using primarily the technique of quail/chick grafting; since species-specific markers can be used to stain quail cells in chick embryos, this method can be used to unambiguously label one population or the other (Narayanan and Narayanan, 1980; D'Amico-Martel and Noden, 1983). These studies have demonstrated that, in the sensory ganglia of cranial nerves VII, IX, and X (geniculate, petrosal, and nodose), the neural crest mainly gives rise to the neurons of the proximal ganglia of the cranial nerves, near the neural tube, whereas the placodes mainly give rise to the neurons of the more ventral distal ganglia. Neural crest cells do contribute to the distal ganglia, but in these studies, they were not observed to form neurons there. Strong expression of CACNG4 is evident in the distal ganglia (Figs. 2C, 2F, and 2G), suggesting that CACNG4 may be expressed by placode-derived cells. However, CACNG4 is also expressed in the DRGs, suggesting that neural crest-derived cells can also express CACNG4.

To determine the origin of CACNG4-expressing cells within the cranial ganglia, we used isotopic grafts of quail tissue into chick embryos to label either the placode-containing ectoderm or the neural crest-containing dorsal neural folds, at stages 8–10, prior to cell migration/invagination. In separate experiments, we also labeled the neural crest or placodes with the lipophilic dye DiI and obtained the same results described below (data not shown.) After 48 h of incubation (at about stage 20), embryos were fixed and sectioned at 6 μm, which is sufficiently thin to allow single-cell analysis. Subsequently, CACNG4 was visualized by *in situ* hybridization (blue stain, Fig. 4), and quail cells were visualized by antibody staining, using the QCPN anti-quail cell antibody (brown stain, Fig. 4).

When placode cells were labeled by grafting of quail ectoderm, large numbers of quail placodal cells contributed to the otic vesicle and acoustic ganglion (Figs. 4A and 4B), and the distal petrosal (data not shown) and nodose ganglia (Figs. 4C and 4D), as demonstrated previously. Within these ganglia, many of the placode-derived cells express CACNG4 (arrowheads, Figs. 4B and 4D). Since placode-derived cells form neurons in the distal ganglia, CACNG4 likely marks neurons which are differentiating at this stage.

When neural crest cells were labeled by grafting of quail neural folds (Figs. 4E–4H), neural crest-derived cells were observed, as expected, primarily in the proximal ganglia (arrow, Fig. 4E), which do not express CACNG4. However, some neural crest cells also contributed to the distal ganglia, including the distal petrosal (Figs. 4E and 4F) and distal nodose (Figs. 4G and 4H). When viewed at high magnification, one can distinguish that not all brown-stained quail cells express CACNG4, but some are definitely double-stained (arrowheads, Figs. 4F and 4H). Thus, there are neural crest-derived cells in the distal ganglia that do express CACNG4, as do placode-derived cells.

Neural-Crest Derived Cells Can Form Neurons in the Distal Ganglia

If neural crest-derived cells form only nonneural support cells in the distal ganglia, as found in previous studies (Narayanan and Narayanan, 1980; D'Amico-Martel and Noden, 1983), our grafting results would then indicate that these support cells express CACNG4. However, the previous studies did not use molecular markers for neurons, and embryos were analyzed at substantially later stages than in our experiments (see Discussion). We therefore undertook the reexamination of the fate of neural crest cells in the distal ganglia, using chick embryos with grafted quail neural folds, grown to stage 20 (as in Fig. 4), and the Hu antibody as a marker of differentiating neurons. Hu strongly stains neurons in the outer layers of the hindbrain and in the distal ganglia (green stain, Fig. 5); within each neuron, Hu stains the cell body but not the nucleus, such that nuclear QCPN staining (red) of quail cells can be clearly distinguished. In agreement with previous results, the majority of QCPN-positive neural crest-derived cells in the distal ganglia are negative for Hu, indicating a nonneuronal identity. However, as in Fig. 4, a few cells were definitively double-stained (arrowheads, Fig. 5), in both the petrosal (Figs. 5A–5D) and nodose (Figs. 5E–5H) ganglia. We conclude that, contrary to previous belief, neural crest-derived cells can indeed form neurons in the distal ganglia, though only a minority of cells adopt this fate.

DISCUSSION

We have demonstrated, for the first time, expression of a calcium channel regulatory subunit in early development. CACNG4 expression is observed first in the anterior neural plate, and later in a variety of neural tissues, including cranial ganglia, DRGs, and discrete regions of the brain. Significantly, these neural tissues are initiating neuronal differentiation at the same stages when CACNG4 expression begins. However, expression of CACNG4 is not limited to differentiating neurons; differentiating myoblasts in the limb buds also express CACNG4, though this is true for only a subset of myoD-positive cells. Within the cranial ganglia, CACNG4 expression is not limited to cells of one lineage, but rather is shared by both placode-derived and neural crest-derived cells. The surprising finding of CACNG4-positive neural crest-derived cells in the distal

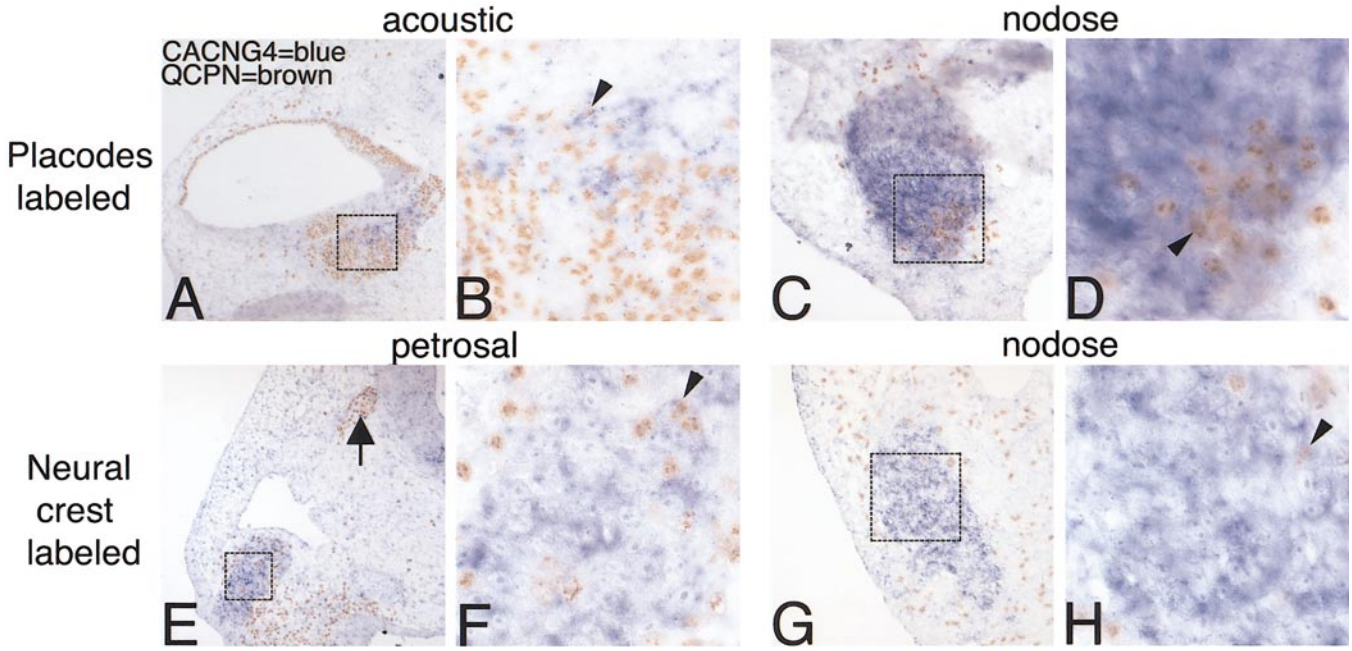

FIG. 4. Expression of CACNG4 in cells of both neural crest and placodal origin. All sections are transverse, through the hindbrain of 3.5-day embryos (stage 20); dorsal is toward the top. CACNG4 expression (blue) was visualized by *in situ* hybridization on thin sections; grafted quail cells (brown) were stained by using the QCPN antibody. Boxes indicate the region enlarged in the subsequent section. (A–D) Ectodermal placode cells were labeled by grafting of quail ectoderm (including also some underlying mesenchymal cells, which do not contribute to the ganglia) into chick embryos. (A) Strong contribution of labeled placodal cells to the otic vesicle and acoustic ganglion (expressing CACNG4). (B) High-magnification view of the acoustic ganglion shown in (A); many labeled quail cells also express CACNG4 (arrowhead). (C) Contribution of placodal cells to the distal nodose ganglion, stained strongly for CACNG4 expression. (D) High-magnification view of the ventral nodose ganglion, showing many double-stained cells (arrowhead). (E–H) Neural crest cells were labeled by grafting of quail neural folds. (E) Contribution of neural crest-derived quail cells to the proximal petrosal ganglion (arrow), but also to the CACNG4-expressing distal ganglion. (F) High-magnification view of the distal petrosal ganglion shown in (E). The arrowhead indicates a quail cell expressing CACNG4. (G) Expression of CACNG4 in the distal nodose ganglion, containing neural crest-derived cells. (H) High-magnification view showing a double-stained cell (arrowhead).

ganglia led us to retest previous assumptions about the fate of neural crest cells in these ganglia; we found that neural crest cells can form neurons there, contrary to previous reports that they only form nonneural support cells in distal ganglia. We conclude that the presence of CACNG4 correlates with the process of differentiation in neurons and myoblasts.

Function of CACNG4

The chick gene we isolated was named based on its strong homology to CACNG4. Mouse CACNG4 has been shown to alter the electrophysiological properties of VDCCs, indicating a function in regulating VDCC activity (Klugbauer *et al.*, 2000). However, CACNG4 bears little resemblance (12.1% similarity) to the γ1 subunit initially isolated based on its association with VDCCs. In contrast, CACNG4 bears a strong similarity to CACNG2, or stargazin. While CACNG2 can likewise alter VDCC kinetics, it has also been proposed to act via AMPA glutamate receptors, which are nonfunctional in the cerebellar granule cells in stargazer mutants (Chen *et al.*, 1999, 2000; Hashimoto *et al.*, 1999). In support of this hypothesis, transfection of γ2 into stargazer mutant neurons is sufficient to rescue AMPA receptor responses, even when VDCCs are blocked (Chen *et al.*, 2000); these studies further suggest that γ2 may act by clustering AMPA receptors at synapses, via a physical association both with AMPA receptor subunits and with the channel-clustering PDZ protein PSD-95. This function has not been tested for CACNG4 homologues, but these genes do share the binding site for PDZ proteins that is present in CACNG2 and required for its clustering/activating activity. In early avian embryos, it is not known whether clustering PDZ proteins, AMPA receptors, or even the other VDCC subunits are present at the same time and in the same tissues as CACNG4. Thus, it remains unclear whether CACNG4 acts primarily in association with VDCCs, or AMPA receptors, or perhaps both.

Despite this controversy over which type of calcium channels is regulated by CACNG4, the net result of either association is likely to be effects on the levels of calcium entering the cell. CACNG4 may therefore be more or less

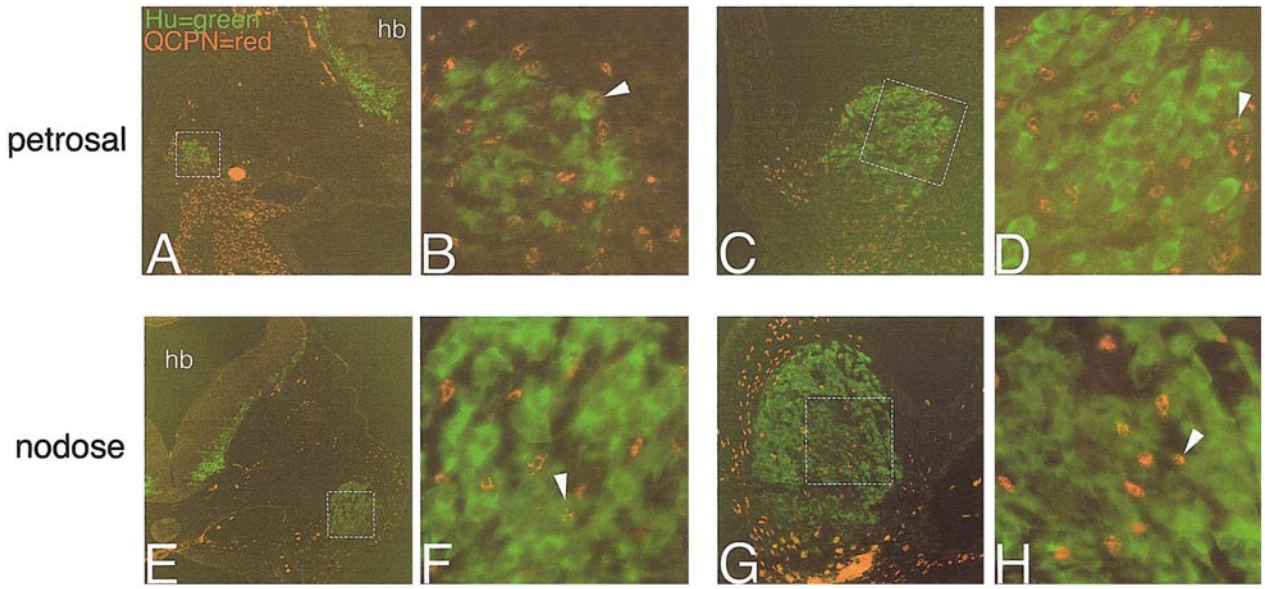

FIG. 5. Some neural crest-derived cells differentiate as neurons in the distal cranial ganglia. Neural crest cells were labeled by grafting of quail neural folds into chick embryos. Quail cell nuclei (red) were stained by using the QCPN antibody; differentiating neurons (green) were stained by using the Hu antibody. All sections are transverse through the hindbrain of stage 20 embryos, with dorsal toward the top. Boxes indicate the region enlarged in the subsequent section. (A) Section through the hindbrain (hb) and distal petrosal ganglion (boxed). (B) High-magnification view showing neural crest contribution to the ganglion. Most quail cells do not express Hu, but one cell (arrowhead) demonstrates clear Hu staining around the quail nucleus (i.e., double-labeled). (C) Section through the petrosal ganglion of a different embryo. (D) High-magnification view showing a double-labeled cell (arrowhead). (E) Section through the distal nodose ganglion. (F) High-magnification view of neurons in the nodose ganglion, including a double-labeled cell (arrowhead). (G) Section through the nodose ganglion of a different embryo. (H) High-magnification view showing a double-labeled cell.

directly involved in generation of the calcium transients observed during development and implicated in multiple developmental processes. Of these processes, CACNG4 expression is most tightly linked to differentiation, in both neural and muscle tissues. CACNG4 need not have a drastic, on-or-off effect in order to affect differentiation; qualitative changes in the type of calcium transient can affect neuronal development dramatically. For example, embryonic *Xenopus* spinal neurons exhibit two types of calcium transients, spikes and waves, with different kinetic properties. Though both produce changes in intracellular calcium levels, the precise kinetics of the change are critical for the effect, as spikes promote neurotransmitter expression and channel maturation, while waves promote neurite outgrowth (Gu and Spitzer, 1995).

We therefore suggest that expression of CACNG4 promotes differentiation. We attempted to test this hypothesis by ectopically expressing CACNG4 throughout the chick neural tube by electroporation; however, we did not observe any effects on the differentiation state of these cells (data not shown). This result is perhaps not surprising in light of the fact that the γ subunit is but one component of calcium channels, and the other components are not known to be present in the affected cells at these stages (electroporated at stages 10–11, with expression beginning about 5 h later). Even if the basic machinery is in place, calcium currents are tightly controlled by a plethora of other factors. CACNG4 may require cofactors not present in these cells in order to alter transients in the precise manner needed to trigger differentiation; alternatively, other regulatory factors may override the effect of CACNG4 in these cells.

While the later expression of CACNG4 correlates with differentiating cell types, CACNG4 is also expressed early in the neural plate, well before differentiation. At these stages, CACNG4 may still act by regulating calcium transients, but the function of these transients may be different. Calcium transients have been implicated in neural induction (Leclerc *et al.*, 2000), but this process is largely complete before the stages examined. Transients have also been suggested to organize large morphogenetic movements (Wallingford *et al.*, 2001); thus, one possibility is that transients may play a role in the movements of neurulation. This model might also explain why CACNG4 is expressed in only the anterior neural plate, where the head folds are actively elevating. Finally, transients may be involved in the signaling pathways acting to pattern the neural plate; the restriction of CACNG4 expression to the anterior neural plate may reflect a role in anteroposterior patterning.

Complexity of CACNG4 Expression Patterns

Initial characterization of the first γ subunits suggested very simple patterns of expression: γ1 in muscle (Jay *et al.*, 1990), and γ2 in neural tissue (Letts *et al.*, 1998). However,

recent analysis by RT-PCR indicated that expression of these and other γ subunits was much more widespread among different organs and tissues (Burgess *et al.*, 2001). Our results, showing CACNG4 expression in both neural and muscle tissue, support this latter idea. Additionally, our detailed *in situ* analysis shows even greater complexity within tissues, such as the expression of CACNG4 in some but not all differentiating myoblasts. This restriction may indicate that CACNG4 acts in only a brief phase of differentiation, in a narrower window than that of myoD.

Although CACNG4 expression was not restricted to cells of one tissue or one lineage, it was limited to excitable cells, and was not expressed in nonexcitable neural crest derivatives (such as melanocytes) or other tissues. However, these nonexpressing tissues may express one or more of the other γ subunits. The expression patterns of these genes have not been examined in early development, but expression of all eight human γ subunits was detected in fetal brain tissue, and CACNG2, -4, and -7 were detected in fetal liver (Burgess *et al.*, 2001). Further expression studies of these genes in chick and mouse embryos are likely to reveal new patterns and new roles for γ subunits in development.

CACNG4 Expression and Neural Crest-Derived Neurons within the Distal Cranial Ganglia

Although cranial ganglia are composed of cells from the neural crest and the ectodermal placodes, these two sources do not contribute equally to all regions within the ganglia. Within the distal ganglia, previous studies found that the neurons are derived from placodes, while the nonneural support cells are derived from neural crest (Narayanan and Narayanan, 1980; D'Amico-Martel and Noden, 1983). As expected, we found that labeling of the placodes produces a large contribution to the distal ganglia, and that many of these prospective differentiating neurons express CACNG4. Surprisingly, we found that some neural crest-derived cells within the distal ganglia also express CACNG4. If these neural crest-derived cells represent prospective glia, as predicted by the prior studies, this observation could represent an exception to the pattern of CACNG4 expression in differentiating tissues, since glial cells do not differentiate until substantially later in development.

However, an alternative possibility is that the few CACNG4-positive neural crest-derived cells do represent differentiating neurons. D'Amico-Martel and Noden (1983) did observe one neural crest-derived neuron in the distal ganglia, indicating that it is possible for neural crest cells to become neurons in this location, even if this is not the norm. Moreover, these earlier studies only examined embryos at much later stages (stages 30–38), perhaps missing neural crest-derived neurons present at the earlier stages examined here. Finally, the previous studies determined neuronal identity based on histological markers, which may not recognize all neurons. Given these concerns, we decided to reexamine this issue at earlier stages and did find that some neural crest cells can form neurons in the distal ganglia. We therefore suggest that these differentiating neurons likely represent the CACNG4-positive population of neural crest-derived cells, though it was not possible to test this directly due to antibody-incompatibility problems.

One remaining question is why neural crest-derived neurons (other than the one noted by D'Amico-Martel and Noden) were not observed in the distal ganglia in the previous studies. Such neurons may have simply been overlooked, given their low frequency, or unstained by the histological markers used to detect neurons. Alternatively, it may be that these cells, perhaps lacking the appropriate growth factors in their inappropriate environment, undergo cell death prior to the late stages examined in the previous reports. Further experiments, with additional stages and markers, will be necessary to characterize the development and survival of these previously undetected neurons.

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